Synthesis of fatty acid hydroperoxide in the presence of organic solvent using immobilized lipoxygenase[1]

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Lipoxygenase (EC 1.13.11.12) catalyses the dioxygenation of polyunsaturated fatty acids, forming their corresponding hydroperoxides. The ability of immobilized lipoxygenase to introduce oxygen derived from air into linoleic acid in a medium containing organic solvent and aqueous buffer was investigated. Reaction medium parameters such as the level of aqueous buffer, buffer pH, type of organic solvent, reaction temperature and lipoxygenase level were altered to test their influence upon the degree of oxygenation. The time course of oxygenation was followed, and the resulting data were analysed by non-linear regression to determine the maximum hydroperoxide that could be generated, as well as the reaction half-time. The results demonstrated that the highest efficiency in hydroperoxide formation occurs when the oxygenation reaction is conducted relatively rapidly, although not so rapidly as to cause the reaction milieu to become anaerobic. In an optimized reaction medium at 15 °C containing 15 ml of water-saturated octane, 6 ml (35%, v/v) 0.2 M borate buffer, pH 9.0, 40 mg of linoleic acid, and immobilized lipoxygenase containing 3.0 mg of protein, a yield of hydroperoxyoctadecadienoic acid greater than 80% was obtained from linoleic acid in 2 h. H.p.l.c. analysis showed that approx. 97% of the product was 13-hydroperoxy-octadeca-9,11(*Z*,*E*)-dienoic acid. The procedure developed is an improvement over those previously published, in that lipoxygenase and the hydroperoxide product are easily separated, only air at atmospheric pressure is needed as the source of oxygen and no surfactant is required.

Soybean (*Glycine max*) lipoxygenase (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a commercially available enzyme that catalyses the introduction of oxygen into polyunsaturated fatty acids containing a 1,4-diene unit. When linoleic acid is the substrate, 13-hydroperoxyoctadeca-9,11(*Z*,*E*)-dienoic acid (HPOD) is the predominant product formed in an

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[3] Abbreviations used: HPOD, hydroperoxyoctadecadienoic acid; IMM-LOX, immobilized lipoxygenase; LA, linoleic acid; LOX, lipoxygenase.

aqueous medium at high pH [1]. Reduction of fatty hydroperoxides gives hydroxylated fatty acids, and these have the potential to replace ricinoleic acid and its derivatives in industrial products [2,3]. Currently ricinoleic acid is obtained from castor oil, a commodity that is imported into the U.S. at the level of 30000 metric tons/year [3]. In addition to industrial potential, fatty acid hydroperoxides and their derivatives have been identified as potent, biologically active agents with important roles in a variety of physiological processes [4,5].

The study of the catalytic mechanism, regioselectivity and stereospecificity of unbound LOX during its action upon linoleic acid (LA), arachidonic acid and other polyunsaturated fatty acids has been the subject of many publications [1]. In contrast, there have been relatively few attempts to use LOX in a synthetic mode where a high yield of hydroperoxide is desired [6–13]. Actual product yields are often far less than quantitative, due to anaerobic recycling resulting from poor oxygen solubility in aqueous media [1,13]. In an attempt to increase hydroperoxide yield, oxygenation of LA was conducted in aqueous buffer under pure oxygen at 405 kPa (4 atm) pressure at 0°C to give an 80% yield of HPOD [7]. Also, a high yield of hydroperoxide can be obtained in an aqueous medium sparged with oxygen at ambient pressure. However, unless antifoam is used, many additions of LOX are necessary, as bubbling through aqueous dispersions of fatty acid leads to foaming, resulting in enzyme denaturation. Using air as the source of oxygen, high yields of HPOD were obtained at room temperature in emulsions containing organic solvent and aqueous buffer, but the addition of a non-ionic surfactant was necessary [12].

Prior work in our laboratory resulted in an improved procedure for the preparation of immobilized LOX (IMM-LOX) [14]. The catalytic stability of IMM-LOX was approx. 10 times greater than that of unbound LOX, and the IMM-LOX could be recycled. In the present study the use of IMM-LOX as a catalyst for the formation of fatty acid hydroperoxides in organic-solvent/aqueous-buffer mixtures was investigated. Various reaction parameters, such as the kind of solvent, water level, buffer pH, reaction temperature, and protein level of IMM-LOX, were optimized to achieve the highest yields of HPOD. The addition of organic solvent to the reaction milieu is beneficial because oxygen is many times more soluble in organic solvent than in water. For example, in iso-octane equilibrated with air the oxygen concentration is 3.5 mM, while that in water is only 0.25 mM [15].

Materials and methods

Materials

Soybean LOX (Lipoxidase, Type 1-B), linoleic acid (LA), protein standard and cumene hydroperoxide were purchased from Sigma (St. Louis, MO, U.S.A.). Reacti-Gel® 6X was purchased from Pierce (Rockford, IL, U.S.A.).

The dye reagent for the protein assay was purchased from Bio-Rad (Richmond, CA, U.S.A.). T.l.c. plates were purchased from Analtech (Newark, DE, U.S.A.). Normal-phase h.p.l.c. was conducted with a Chrompack Chromsper SI column (200 mm × 3 mm) installed on a Hewlett–Packard Series 1035 instrument. The sodium salt of Xylenol Orange was purchased from Aldrich (Milwaukee, WI, U.S.A.). Water was purified to a resistance of 18 MΩ·cm using a Barnstead NANOpure system. All other reagents were of the highest purity available.

LOX immobilization

IMM-LOX was prepared using a carbonyldiimidazole-activated matrix termed Reacti-Gel® as described previously [14]. IMM-LOX was stored at 4 °C in 0.1 M phosphate buffer, pH 7.0, containing 0.9% NaCl, 0.05% BSA and 0.02% NaN_3.

HPOD formation

In a typical procedure, 40 mg of LA was dissolved in 15.0 ml of water-saturated octane in a 125 ml glass-stoppered Erlenmeyer flask. After washing IMM-LOX with a 0.2 M borate buffer (pH 9.0), 0.3 g of IMM-LOX containing 1.5 mg of protein and 6 ml of 0.2 M borate buffer, pH 9.0, were added to the solution of LA in water-saturated octane. The reaction was allowed to proceed at 15 °C while the mixture was agitated at 250 rev./min. After a specified reaction time, the gel was filtered from the reaction mixture. The pH of the water layer was lowered to 3.0, and the aqueous and organic layers were separated. The aqueous layer was extracted with 15 ml diethyl ether, then with two 10 ml aliquots of diethyl ether. The organic fractions were combined and an aliquot was withdrawn for HPOD assay.

HPOD assay

HPOD levels were estimated spectrophotometrically using the Xylenol Orange technique [16]. The Xylenol Orange reagent was composed of 100 μM Xylenol Orange, 250 μM ammonium $FeSO_4$, 25 mM H_2SO_4, and 4 M 2,6-di-t-4-methylphenol in methanol water (90:10, v/v). The reagent (2.0 ml) was added to the sample (10–50 μl), and the volume was raised to 2.1 ml with ethanol. The assays were incubated at room temperature for 45 min, and the absorbance at 560 nm was measured versus a blank that was a mixture of 2.0 ml of the Xylenol Orange reagent and 100 μl of ethanol. Freshly diluted commercial cumene hydroperoxide was used for preparing a calibration curve of the dye reagent each day.

A t.l.c. analysis was performed on each reaction mixture as a check on the hydroperoxide levels given by the Xylenol Orange method and also to determine if anaerobic by-product formation and/or decomposition of HPOD was occurring. Silica-gel preadsorbent HPTLC-HL TLC plates (10 cm × 10 cm coating thickness 150 μm) were dipped in 5% boric acid in methanol and allowed to air dry prior to spotting. The t.l.c. plates were developed sequentially in the following solvent systems: diethyl ether/

benzene/ethanol/acetic acid, 200:250:5:1, by vol.; air drying; iso-octane/diethyl ether/acetic acid (25:25:1, by vol.) [17]. Fatty acids were visualized by charring after spraying the t.l.c. plates with 60% H_2SO_4. The R_F values of LA, HPOD, and hydroxyoctadecenoic (ricinoleic) acid were 0.77, 0.62, and 0.53 respectively. Since this t.l.c. system cannot differentiate between fatty acids having differing degrees of unsaturation, the R_F value of hydroxyoctadecadienoic acid, the reduced analogue of HPOD, was also 0.53.

Determination of the regioselectivity of oxygen incorporation

IMM-LOX (0.244 g, containing 1.17 mg of protein) was added to a mixture of 15 ml of hexane and 6 ml of pH 9.0 buffer containing 0.1 M Hepes, Tricine and 2-amino-2-methylpropan-1-ol hydrochloride. After shaking the mixture at 15 °C for 30 min, oxygenation was initiated by the addition of 40 mg of linoleic acid dissolved in 100 μl of ethanol. After 2 h of agitation at 250 rev./min, the pH of the reaction medium was lowered to 3.0. HPOD was extracted with 50 and 25 ml aliquots of diethyl ether. The ether aliquots were combined, dried over anhydrous Na_2SO_4 and evaporated under a stream of nitrogen. HPOD was dissolved in 5 ml of ethanol and reduced with $NaBH_4$ at 0 °C for 30 min, after which the reduced HPOD solution was left at room temperature for 30 min. Water (5 ml) was added, and the pH was adjusted to 3.0 with 1 M HCl. Reduced HPOD was extracted with 50 and 25 ml aliquots of diethyl ether. The combined ether aliquots were washed with 10 ml of water, and their volume was reduced to 2 ml under a stream of nitrogen. Diazomethane in cyclohexane was added to afford the methylated product. The distribution of products into the oxo derivative and the various geometrical isomers of the hydroxy derivative was determined with normal-phase h.p.l.c. on silica gel. The mobile phase consisted of hexane and propan-2-ol, in the following respective proportions: initially, 99.3/0.7; after 20 min, 98.5/1.5; after 25 min, 98.5/1.5. Product levels were determined with an ELSA IIA evaporative light-scattering detector (Varex; Burtonsville, MD, U.S.A.) set at 110 °C.

Time-course analyses

Time courses of HPOD formation were analysed by non-linear regression using the program Abacus, which is based upon the Gauss–Newton iterative method [18]. Iterative analysis was carried out until the minimum in the root-mean-square error was found. The mathematic expression that describes the time course of product formation is as follows:

$$P_{obs.} = \frac{P_{max.} k_1^n t^n}{1 + k_1^n t^t} \quad (1)$$

where $P_{obs.}$ is the amount of product observed at time t, $P_{max.}$ is the maximum amount of product formed, k_1 is the rate of reaction at one-half $P_{max.}$, and n is the co-operativity parameter that is constrained to integer

values. The reciprocal of k_1 yields the time needed to generate one-half $P_{max.}$ and is therefore the reaction half-time.

Results and discussion

Figure 1 shows the effect of the addition of 0.2 M borate buffer, pH 9.0, upon the activity of IMM-LOX in octane at 15 °C. The octane had been previously saturated with water, and therefore a significant amount of HPOD formed even when no buffer was added. By increasing the amount of added buffer to about 35% (v/v), the rate of HPOD formation could be increased by more than 3-fold.

Figure 2 shows the influence of changing buffer pH upon the activity of IMM-LOX in an octane/aqueous-buffer mixture. The highest levels of HPOD biosynthesis took place at approx. pH 9.0. The activity of IMM-LOX decreased at lower pH values; the rate at pH 7.0 was only about one-sixth as great as it was at pH 9.0. Raising the pH of the aqueous buffer above pH 9.0 resulted in a modest decrease in the amount of HPOD formed.

The rate of oxidation of LA catalysed by IMM-LOX was determined when aqueous buffer was added to different organic solvents (Table 1). Water-saturated solvents were used to ensure that the level of free water was constant as the solvents were varied. The highest amounts of HPOD were obtained with 1,1,2-trichlorotrifluoroethane. Approximately equal amounts of HPOD were formed when the solvents hexane, heptane, octane and 2,2,4-trimethylpentane were added, but the amount of HPOD was slightly reduced from that formed in 1,1,2-trichlorotrifluoroethane. Use of toluene and cyclohexane resulted in a further decrease in the amount of HPOD formed. The lowest detectable levels of HPOD were found in reaction mixtures that contained diethyl ether and di-isopropyl ether. HPOD formation was not detected in reactions conducted with butanone or octanone. That the ethers and ketones were found to inhibit the action of IMM-LOX is consistent with other work showing that, generally, polar organic solvents are detrimental to enzymic activity [19].

The time course of HPOD formation was examined over a 24 h period at three different temperatures (7, 15 and 25 °C) in an octane/borate buffer mixture using IMM-LOX containing 0.25 mg of protein. The data were fitted by non-linear regression analyses to eqn. (1), and the resulting progress curves revealed $P_{max.}$, the maximum amount of HPOD that can be formed and $t_{\frac{1}{2}}$, the reaction half-time (Table 2) [18]. All curve-fits were performed with n equal to 1 (no co-operativity) and n equal to two (co-operativity). Comparison of the calculated root-mean-square errors and errors of the coefficients revealed the best fits of the calculated curves to the data were obtained without evoking co-operativity. Although $t_{\frac{1}{2}}$ decreased 5-fold as the temperature increased from 7 to 25 °C, $P_{max.}$ also decreased, and the calculated percentage yield of HPOD

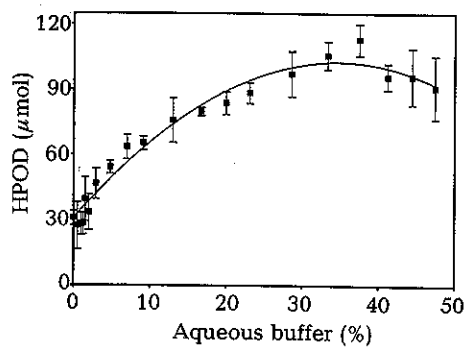

Figure 1

Influence of the amount of aq. 0.2 M borate buffer, pH 9.0, on HPOD formation catalysed by IMM-LOX. In addition to the buffer, each assay mixture contained 0.75 g IMM-LOX containing 3.0 mg of bound protein, 40 mg of linoleic acid and 15 ml of water-saturated octane. Assays were conducted for 3 h at 15 °C. The percentage buffer values were calculated as ml of buffer/(ml of buffer + ml of octane) × 100. The data are means ± S.E.M. for ten determinations.

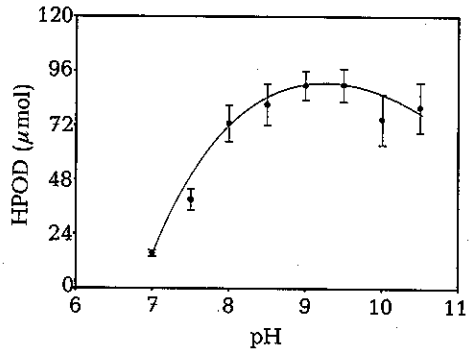

Figure 2

Influence of buffer pH upon HPOD formation catalysed by IMM-LOX. The assays contained 6 ml of buffer containing a mixture of 0.1 M Hepes, Tricine, and 2-amino-2-methylpropan-1-ol, hydrochloride, 15 ml of water-saturated octane, 0.67 g of IMM-LOX containing 3 mg of bound protein and 40 mg of linoleic acid. The assays were conducted for 3 h at 15 °C. The data are means ± S.E.M. for six determinations.

Table 1 Influence of organic solvent upon HPOD formation catalyzed by IMM-LOX
Each assay contained 15 ml of water-saturated organic solvent, 6 ml of 0.2 M borate buffer, pH 9.0, 0.194 g of IMM-LOX containing 1 mg of bound protein and 40 mg of linoleic acid. The assays were conducted for 1 h at 15 °C. Results are means ± S.E.M. for three repetitions

Organic solvent	*HPOD (μmol)*
1,1,2-Trichlorotrifluoroethane	22.2 ± 1.2
Heptane	17.9 ± 1.1
Hexane	17.7 ± 1.4
2,2,4-Trimethylpentane	16.6 ± 0.5
Octane	16.0 ± 1.8
Toluene	10.1 ± 1.1
Cyclohexane	8.9 ± 0.6
Diethyl ether	4.0 ± 0.3
Isopropyl ether	2.3 ± 0.3

fell from 81 to 38%. Analysis of the reaction products obtained at 25 °C by t.l.c. revealed that, in addition to HPOD, substantial by-product formation had occurred, as evidenced by materials that had lower R_F values than that of HPOD.

The effect of increasing amounts of IMM-LOX upon HPOD formation at 15 °C was investigated to determine if the yield of HPOD could be increased, or whether its yield was limited by the concentration of

Table 2 Non-linear regression analysis of the progress curves of HPOD formation by IMM-LOX at three temperatures in octane/borate buffer media

IMM-LOX[a] (mg of protein)	Temperature (°C)	n[b]	P_{max}[c] (*μmol*)	*HPOD yield* (%)	$t_{1/2}$[d] (*h*)
0.25	7	1	115±7[e]	81±5	16.8±20
0.25	15	1	98±2	69±2	11.3±0.6
0.25	25	1	54±2	38±1	3.3±0.3
3.0	15	1	152±8	106±6	1.1±0.2
3.0	15	2	119±4	84±3	0.70±0.06

[a] There were 5.09 mg of protein/g of IMM-LOX.
[b] n is the co-operativity parameter.
[c] P_{max} is the maximum amount of HPOD formed.
[d] $t_{1/2}$ is the reaction half-time.
[e] The listed error is the asymptotic S.E.M., an estimate of the S.D. in the estimate of the parameter.

oxygen in the reaction medium. As shown in Figure 3, the yield of HPOD was greatly increased as the protein bound to IMM-LOX was increased from 0.25 to 5.0 mg. Most of the increase in the HPOD yield occurred as the level of protein was raised up to 3.0 mg, with relatively little enhancement observed above this protein level, probably because oxygen transfer to the reaction medium became rate-limiting.

The time course of HPOD formation was examined with non-linear regression analysis over a 6 h period at 15 °C using IMM-LOX that contained 3.0 mg of protein, a 12-fold increase in protein over that used previously (Table 2). Curve-fit estimates were again obtained with the co-operativity parameter, n, equal to 1 and 2. That a co-operative model ($n=2$) provided the best fit of the data was demonstrated by several observations. First, t.l.c. analysis showed that a portion of LA was converted into by-products as indicated by materials that had lower R_F values than that of HPOD (Figure 4). Thus a potential HPOD yield of about 100%, as was given when n was set equal to 1, is not realistic, while when n was equal to 2, the yield was 84%. Secondly, when the value of n was set equal to 2, the calculated reaction half time, $t_{\frac{1}{2}}$, was 42 min, which closely corresponds to that indicated by visual observation of the t.l.c. plate. Finally, when n was increased from 1 to 2, the errors of the coefficients decreased.

After oxygenation of LA by IMM-LOX in the presence of organic solvent, the products were reduced to their corresponding hydroxy derivatives, methylated and analysed by h.p.l.c. Five products were resolved. The peak corresponding to oxo fatty acid was identified by its distinctive absorbance at 280 nm [20]. The remaining peaks were assigned to the various double-bond and hydroxyl-group isomers of reduced HPOD using prior investigations as a guide [21]. These assignments were confirmed by analysing the products derived from oxygenations conducted in all aqueous media at pH 9.0 and 7.0. It is known that, at pH 9.0, the predominant isomer of HPOD formed is 13-hydroperoxy-octadeca-9,11(*Z*,*E*)-dienoic acid, while at pH 7.0 the amount of the 13-

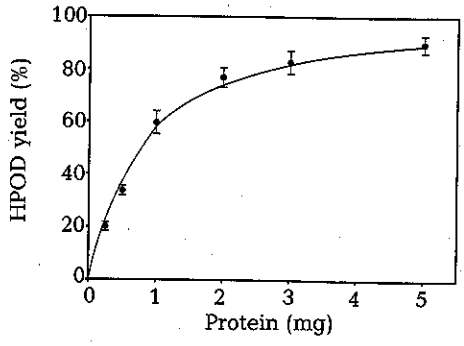

Figure 3

Influence of the amount of IMM-LOX on the percentage yield of HPOD in octane/borate buffer at 15 °C. Each assay mixture contained 15 ml of water-saturated octane, 6 ml of 0.2 M borate buffer, pH 9.0, 40 mg of linoleic acid and the indicated amount of IMM-LOX (5.09 mg of protein/g of gel). The assays were conducted for 4 h. Results are the mean values ± S.E.M. for four determinations.

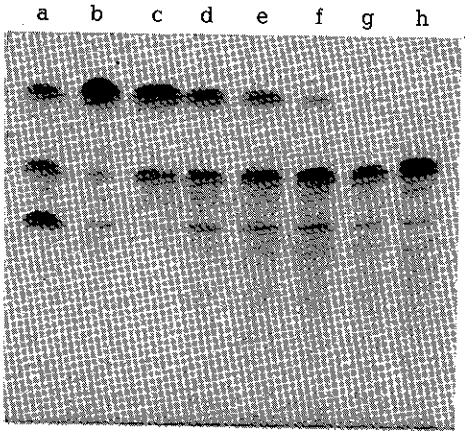

Figure 4

A typical time course of HPOD formation as analysed by t.l.c. The assay mixtures contained IMM-LOX containing 3 mg of protein and the levels of octane, borate buffer and linoleic acid described in Figure 3. Lane a, standards: top, LA; middle, HPOD; bottom, ricinoleic acid; Lanes b–h, assays quenched after 15, 30, 45, 60, 90, 120, and 180 min.

isomer decreases, and the amount of 9-hydroperoxyoctadeca-10,12(*E*,*Z*)-dienoic acid increases [22]. Analyses of the reaction products obtained from media containing hexane and pH 9.0 aqueous buffer demonstrated that the predominate isomer synthesized by IMM-LOX is 13-hydroperoxyoctadeca-9,11(*Z*,*E*)-dienoic acid. The percentage of total product of this isomer was 96.7 ± 1.4% (S.E.M., $n = 3$) as determined by a Varex evaporative light-scattering detector. In a prior study of soybean LOX in reverse micelles of sodium bis-(2-ethylhexyl)sulphosuccinate in the presence of n-octane, 13-hydroperoxyoctadeca-9,11(*Z*,*E*)-dienoic acid was also found to be the predominant isomer synthesized [23].

Conclusions

HPOD yields of greater than 80% can be obtained in mixture of organic solvent and aqueous buffer using IMM-LOX as the catalyst. This yield is as high as that obtained previously using pure oxygen, rather than air [7,9], although somewhat lower than that obtained in the presence of both organic solvent and surfactant [12]. However, the present procedure

avoids the difficult problem of separating the surfactant and the product. It is important to recognize that the fatty acid anion of HPOD itself acts a a surfactant in this system, and that the emulsion which is obtained is extremely stable, being broken only after the reaction medium is acidified, resulting in the protonation of the carboxylate function of HPOD.

The enzyme efficiency factor (*EEF*) is described by the following expression:

$$EFF = \frac{\text{HPOD yield}}{t_{\frac{1}{2}}} \tag{2}$$

The *EEF* value calculated from eqn. (2) with the data that was obtained from the low level of IMM-LOX (0.25 mg of protein) at 15 °C is 6.1 ± 0.3, while that obtained from the high level of IMM-LOX (3.0 mg of protein) at 15 °C is 120 ± 9, a 19-fold increase. This is a greater increase than is expected from the 12-fold increase in added protein. Thus the results show that, as long as anaerobic conditions are avoided, HPOD formation should be conducted as rapidly as possible to achieve the highest efficiency in the production of HPOD.

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